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BARD Project Number: US-3551-04

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Project Title: Embryo transfer as a tool for improving fertility of heat-stressed dairy cattle

Investigators	**Institutions**
Principal Investigator (PI):	
P.J. Hansen	University of Florida
Co-Principal Investigator (Co-PI):	
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Collaborating Investigators: Christine Wrenzycki and Heiner Niemann (Institute for Animal Science, Department of Biotechnology, Neustadt, Germany)

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
In vitro produced embryos, insulin-like growth factor-1, apoptosis, cryopreservation

Abbreviations commonly used in the report, in alphabetical order**:** CCCP, carbonyl cyanide 3-chlorophenylhydrazone**;** IGF-1, insulin-like growth factor-1

Budget: IS: $ 166,000 US: $ 164,000 Total: $ 330,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers) (US only)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		9		
Submitted, in review, in preparation		2		
Invited review papers		3		
Book chapters				
Books				
Master theses		1		
Ph.D. theses		3		
Abstracts		10		
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.
None

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1			1
Longer Visits (Sabbaticals)				0

Description Cooperation:
This project represents a *synergistic* collaboration between Drs. Arav and Hansen. The laboratory at Volcani Center has become one of the leading laboratories in the world in developing novel procedures for cryopreservation and for application of robotics and remote sensing technology to embryo production. One limitation in developing this research to its full potential has been the limited ability to perform embryo transfer on large numbers of cows to verify the applicability of these new technologies under on-farm conditions. Dr. Hansen has been leading an effort to develop embryo transfer as a method for increasing pregnancy rate in heat-stressed dairy cattle. While results indicate the efficacy such an approach, the lack of a suitable cryopreservation system has limited the penetration of embryo transfer into commercial herds. The collaboration will provide Dr. Arav with the capacity to perform large-scale embryo transfer studies while allowing Dr. Hansen to incorporate cryopreservation into the system for using embryo transfer to improve pregnancy rates during heat stress. To date, opportunities to utilize novel cryopreservation procedures developed in Israel for embryo transfer trials in Florida have

not been realized but it is anticipated that completion of the work in Israel will be followed up with such transfer experiments.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				0
Licensed				0

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Abstract

The overall objective of the current proposal is to develop procedures to improve the pregnancy rate achieved following transfer of fresh or cryopreserved embryos produced in the laboratory into heat-stress recipients. The overall hypothesis is that pregnancy rate in heat-stressed lactating cows can be improved by use of embryo transfer and that additional gains in pregnancy rate can be achieved through development of procedures to cryopreserve embryos, select embryos most likely to establish and maintain pregnancy after transfer, and to enhance embryo competence for post-transfer survival through manipulation of culture conditions. The original specific objectives were to 1) optimize procedures for cryopreservation (Israel/US), 2) develop procedures for identifying embryos with the greatest potential for development and survival using the remote monitoring system called EmbryoGuard (Israel), 3) perform field trials to test the efficacy of cryopreservation and the EmbryoGuard selection system for improving pregnancy rates in heat-stressed, lactating cows (US/Israel), 4) test whether selection of fresh or frozen-thawed blastocysts based on measurement of group II caspase activity is an effective means of increasing survival after cryopreservation and post-transfer pregnancy rate (US), and 5) identify genes in blastocysts induced by insulin-like growth factor-1 (IGF-1) (US). In addition to these objectives, additional work was carried out to determine additional cellular determinants of embryonic resistance to heat shock. There were several major achievements. Results of one experiment indicated that survival of embryos to freezing could be improved by treating embryos with cytochalasin B to disrupt the cytoskeleton. An additional improvement in the efficacy of embryo transfer for achieving pregnancy in heat-stressed cows follows from the finding that IGF-1 can improve post-transfer survival of in vitro produced embryos in the summer but not winter. Expression of several genes in the blastocyst was regulated by IGF-1 including IGF binding protein-3, desmocollin II, Na/K ATPase, Bax, heat shock protein 70 and IGF-1 receptor. These genes are likely candidates 1) for developing assays for selection of embryos for transfer and 2) as marker genes for improving culture conditions for embryo production. The fact that IGF-1 improved survival of embryos in heat-stressed recipients only is consistent with the hypothesis that IGF-1 confers cellular thermotolerance to bovine embryos. Other experiments confirmed this action of IGF-1. One action of IGF-1, the ability to block heat-shock induced apoptosis, was shown to be mediated through activation of the phosphatidylinositol 3-kinase pathway. Other cellular determinants of resistance of embryos to elevated temperature were identified including redox status of the embryo and the ceramide signaling pathway. Developmental changes in embryonic apoptosis responses in response to heat shock were described and found to include alterations in the capacity of the embryo to undergo caspase-9 and caspase-3 activation as well as events downstream from caspase-3 activation. With the exception of IGF-1, other possible treatments to improve pregnancy rate to embryo transfer were not effective including selection of embryos for caspase activity, treatment of recipients with GnRH.and bilateral transfer of twin embryos. In conclusion, accomplishments achieved during the grant period have resulted in methods for improving post-transfer survival of in vitro produced embryos transferred into heat-stressed cows and have lead to additional avenues for research to increase embryo resistance to elevated temperature and improve survival to cryopreservation. In addition, embryo transfer of vitrified IVF embryos increased significantly the pregnancy rate in repeated breeder cows.

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Significance of main scientific achievements or innovations

The overall objective was to develop procedures to improve the pregnancy rate achieved following transfer of fresh or cryopreserved embryos produced in the laboratory into heat-stress recipients. It was hypothesized that pregnancy rate in heat-stressed lactating cows can be improved by use of embryo transfer and that additional gains in pregnancy rate can be achieved through development of procedures to cryopreserve embryos, select embryos most likely to establish and maintain pregnancy after transfer, and to enhance embryo competence for post-transfer survival through manipulation of culture conditions. The following narrative highlights some major accomplishments.

Enhancement of cryopreservation. One limitation to the use of in vitro–produced embryos in cattle is poor survival following cryopreservation. Two approaches for enhancing survival of in vitro–produced embryos following cryopreservation were evaluated: culture in the presence of hyaluronic acid and alterations in the cytoskeleton through treatment with cytochalasin B treatment before cryopreservation. Cytochalasin B increased the percent of embryos that re-expanded and that hatched following thawing. The hatching percent was 29.6% for embryos treated with cytochalasin B versus 9.1% for control embryos. There was no significant effect of hyaluronic acid on survival.

Embryo transfer as a mean of increasing pregnancy rates in repeat breeder cows

In the additional year of the BARD project, we transferred in Israel either in vitro fertilization (IVF) embryos or parthenogenic embryos and evaluated the cows 35 days after artificial insemination (AI). Only repeat breeder (RB) (between 3 and 6 AI) were chosen for this study. Primiparous (n=7) and multiparous (n=37). Holstein cows were selected for the experiment between April and June of 2008. Slaughter house ovaries were collected during the winter of the same year, the ovaries were brought to the laboratory and oocytes were aspirated and in-vitro matured for 24h. IVF was done with sperm from the same bull and chemical activation was done with ionomycin and 6 DMAP. Fertilized embryos and parthenogenic embryos were cultured for 1 week until they reached the blastocyst stage. Day 7 embryos were vitrified using ethylene glycol and trehalose and cooled using the VitMaster at ultra rapid cooling rate. The cryopreserved embryos were warmed prior to embryo transfer (ET) on location at the dairy farm one week after natural heat and AI was performed. After palpation, two parthenogenic embryos or one IVF embryo were transferred to the ipsilateral or contralateral corn, respectively. We checked with ultrasound the present of pregnancy 35 days after AI. The overall Pregnancy (PR) rate in this farm was 33% for multiparous (n=144) and 37% for primiparous (n=35). And for RB without ET (control) results were 19%, 30%, 15% and 35% for 3,4,5,6 AI, respectively with an overall of 24% of PR. Results with ET showed increasing PR between 3, 4, 5 and 6 inseminations: 31%, 23%, 50% and 33%, respectively with an overall PR of 34% (15/44) which is 10% higher then control. The PR for the parthenogenic and IVF embryos was 27% and 50% respectively. A paternity test (DNA) of the calves born from the ET shows that all were born (n=7) from the AI and not from the ET-IVF embryos. We conclude that ET of one week following AI can increase the PR of repeat breeder cows.

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Actions of IGF-1 on preimplantation bovine embryos. Earlier results indicated that pregnancy rate of lactating cows used as embryo transfer recipients during summer was higher if cows received embryos cultured with IGF-1 than if cows received embryos cultured with control embryos. For the current proposal, an experiment was conducted to determine whether the effect of IGF-1 on post-transfer embryo survival was a general effect or one specific to heat stress. Lactating recipients (n = 311) were synchronized for timed-embryo transfer. Embryos produced in vitro were cultured $\pm$ 100 ng/ml IGF-1. Recipients receiving IGF-1 treated embryos had higher pregnancy rates at day 45 of gestation in the hot season (42% pregnant for IGF-1 vs 18% pregnant for control) but not in the cool season (23% vs 28%). Results indicate that treatment of embryos with IGF-1 can improve pregnancy rates following the transfer of in vitro produced embryos into lactating recipients, but only under heat-stress conditions.

Expression of several genes in the blastocyst was regulated by IGF-1 including IGF binding protein-3, desmocollin II, Na/K ATPase, Bax, heat shock protein 70 and IGF-1 receptor. These genes are likely candidates 1) for developing assays for selection of embryos for transfer and 2) as marker genes for improving embryo culture conditions.

The fact that IGF-1 improved survival of embryos in heat-stressed recipients only is consistent with the hypothesis that IGF-1 confers cellular thermotolerance to bovine embryos. Other experiments confirmed this action of IGF-1. One action of IGF-1, the ability to block heat-shock induced apoptosis, was shown to be mediated through activation of the phosphatidylinositol 3-kinase/Akt pathway. Effects of heat shock (41^{o}C for 15 h) on induction of apoptosis and reduction in cell number in bovine embryos collected at Day 5 after fertilization were blocked by addition of 100 ng/ml IGF-I at the initiation of heat shock. The IGF-I induced block to apoptosis was eliminated if embryos were cultured with a phosphatidylinositol 3-kinase inhibitor or an Akt inhibitor.

Identification of other cellular determinants of resistance of embryos to elevated temperature. Identification of physiological conditions that alter embryonic survival following heat shock could result in novel strategies for enhancing fertility of heat-stressed cows. One such determinant of embryonic survival to heat shock is redox status. It was found that the actions of heat shock to reduce embryonic development and to increase incidence of apoptosis in embryonic blastomeres was blocked if embryos were cultured in a low oxygen environment.

Depending upon its extent, apoptosis can either cause embryonic death or participate in the process by which embryos recover from deleterious actions of heat shock on development. The ceramide signaling pathway was found to be an important regulator of apoptosis in the embryo. Treatment of embryos > 16 cells collected at Day 5 after insemination with 50 μM C(2)-ceramide increased caspase-9 activity and the proportion of blastomeres undergoing apoptosis. That heat shock may activate ceramide signaling was indicated by findings that exposure of Day 5 embryos to 41^{o}C for 15 h increased activity of the enzyme neutral sphingomyelinase that cleaves sphongomyelin to ceramide.

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The capacity of the preimplantation embryo to undergo apoptosis in response to external stimuli is developmentally regulated. Acquisition of apoptosis does not occur in the cow embryo until between the eight- and sixteen-cell stage. Experiments were performed to delineate the mechanism by which apoptosis is blocked in the bovine two-cell embryo. Results indicate that heat shock does not increase caspase-9 and group II caspase activity in the two-cell embryo. Furthermore, actions of group II-caspases to activate DNases are inhibited at this stage in development.

Agricultural and/or economic impacts of the research findings

The immediate impact is the demonstration that IGF-1 has particular actions on bovine preimplantation embryos such that pregnancy rates in lactating recipients are increased following transfer of IGF-1 treated embryos during heat stress only. Thus, one can improve pregnancy rates in the summer, but not winter, by transfer of embryos cultured with IGF-1. In the long term, incorporation of cytochalasin B into cryopreservation procedures may enhance post-transfer survival of cryopreserved embryos. Moreover, identification of IGF-1 induced genes in the blastocyst may lead to development of markers of embryonic competence for post-transfer survival. Elucidation of the pathways through which IGF-1 causes embryonic thermotolerance as well as identification of other determinants of embryonic resistance to heat shock could lead to new therapeutic approaches for improving fertility during heat stress.

Details of cooperation

To date, collaboration has been consultative only. Following development of improved procedures for cryopreservation of embryos by the Volcani group, however, synergistic collaboration will result. In particular, the effectiveness of the cryopreservation procedure developed in Israel will be tested in Florida using the large commercial dairy herds available to Dr. Hansen.

List of Publications

Refereed publications

Yavin S., Arav A., Measurement of essential physical properties of vitrification solutions. Theriogenology. 2007 Jan 1;67(1):81-9. Epub 2006 Oct 27.

Arav A, Zvi R. Do chilling injury and heat stress share the same mechanism of injury in oocytes? Mol Cell Endocrinol. 2008 Jan 30;282(1-2):150-2.

Arav A, Aroyo A, Yavin S, Roth Z.
Prediction of embryonic developmental competence by time-lapse observation and 'shortest-half' analysis. Reprod Biomed Online. 2008 Nov;17(5):669-75

Yavin S, Aroyo A, Roth Z, Arav A.
Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush Hum Reprod. 2009 Jan 12

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Block, J., and Hansen, P.J. (2007) Interaction between season and culture with insulin-like growth factor-1 on survival of in-vitro produced embryos following transfer to lactating dairy cows. *Theriogenology* **67**, 1518-1529.

Block, J., Fischer-Brown, A.E., Rodina, T.M., Ealy, A.E., and Hansen, P.J. (2007) The effect of in vitro treatment of bovine embryos with IGF-1 on subsequent development in utero to Day 14 of gestation. *Theriogenology*, **68**, 153-161.

Block, J., Wrenzycki, C., Niemann, H., Herrmann, D., and Hansen, P.J. (2007) Effects of insulin-like growth factor-1 on cellular and molecular characteristics of bovine blastocysts produced in vitro. *Mol. Reprod. Dev.*, in press.

Brad, A.M., and Hansen, P.J. The block to apoptosis in bovine two-cell embryos involves inhibition of caspase-9 activation and caspase-mediated DNA damage. *Reproduction*, in press.

de Castro e Paula, L.A. and Hansen, P.J. (2007) Interactions between oxygen concentration and glucose concentration that modulate actions of heat shock on bovine oocytes during in vitro maturation. *Theriogenology* **68**, 763-770.

de Castro e Paula, L.A., and Hansen, P.J. (2007) Ceramide inhibits development and cytokinesis and induces apoptosis in preimplantation bovine embryos. *Mol. Reprod. Dev.*, in press.

de Castro e Paula, L.A., and Hansen, P.J. Role of oxygen and oxidative stress in actions of heat shock on development and apoptosis of preimplantation bovine embryos. *Mol. Reprod. Dev.. Reprod.*, submitted.

de Castro e Paula, L.A., Andrzejewski, J., Julian, D., Spicer, L.J., and Hansen, P.J. Effect of heat stress on oxygen and steroid concentrations in preovulatory follicles of lactating cows exposed to acute heat stress. *Theriogenology*, submitted.

Franco, M., and Hansen, P.J. (2006) Effects of hyaluronic acid in culture and cytochalasin B treatment before freezing on survival of cryopreserved bovine embryos produced in vitro. *In Vitro Cell Dev. Biol. Anim.* **42**, 40-44.

Franco, M., Block, J., Jousan, F.D., de Castro e Paula, L.A., Brad, A., Franco, J.M., Grisel, F., Monson, R.L., Rutledge, J.J., and Hansen, P.J. (2006) Effect of transfer of one or two embryos and administration of gonadotropin releasing hormone on pregnancy rates of heat-stressed dairy cattle. *Theriogenology*, **66**, 224-233.

Jousan, F.D., Oliveira, L.J., and Hansen, P.J. (2007) Short-term culture of bovine preimplantation embryos with insulin-like growth factor-I prevents heat shock-induced apoptosis through activation of the phosphatidyl 3-kinase/Akt pathway. *Mol. Reprod. Dev.*, in press.

Loureiro, B., Brad, A.M., and Hansen, P.J. (2007) Heat shock and tumor necrosis factor-α induce apoptosis in bovine preimplantation embryos through a caspase-9 dependent mechanism *Reproduction* **133**, 1129-1137.

Review Article (Refereed)

Hansen, P.J. (2007) To be or not to be - determinants of embryonic survival following heat shock. *Theriogenology*, **68S**, S40-S48.

Hansen, P.J. (2007) Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. *Theriogenology*, **68S**, S242-S249.

Review Article (Non-refereed)

Hansen, P.J. (2006) Embryo transfer as a tool for fertility enhancement of dairy cattle in hot climates. *Acta Sci. Vet.*, **34** (Suppl. 1), 145-157 (also in Portuguese, same citation).

Theses and Dissertations

Block, J. (2007) *Effect of Insulin-Like Growth Factor-1 on Development and Post Transfer Survival of Bovine Embryos Produced In-Vitro.* Ph.D. Dissertation, University of Florida, Gainesville.

de Castro e Paula, L.A. (2007) *Role of Oxygen and Sphingomyelin Metabolism in Actions of Heat Shock on The Oocyte and Embryo.* Ph.D. Dissertation, University of Florida, Gainesville.

Franco, M.C. (2005). *Strategies To Enhance Fertility in Dairy Cattle During Summer Including Use Of Cryopreservation of In Vitro Produced Embryos.* M.S. Thesis, University of Florida, Gainesville.

Jousan, F.D. (2006) *Insulin-like Growth Factor-I and Apoptosis as Determinants of Preimplantation Bovine Embryonic Development*. PhD Dissertation, University of Florida, Gainesville.

Abstracts

Block, J., and Hansen, P.J. (2006). Effect of the addition of insulin-like growth factor-1 to embryo culture medium on pregnancy rates following timed embryo transfer in lactating dairy cows. *J. Dairy Sci.* **89** (Suppl. 1), 287.

Block, J., Wrenzycki, C., Herrmann, D., Rodina, T.R., Niemann, H., Ealy, A.D., Fischer-Brown, A.E., and Hansen, P.J. (2007). Effect of insulin-like growth factor-1 during culture on blastocyst mRNA abundance and survival in utero to day 14 of bovine embryos produced in vitro. *J. Dairy. Sci.* **90,** 531.

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de Castro e Paula, L.A., and Hansen, P.J. (2007). Protective effects of dithiothreitol on preimplantation bovine embryos exposed to heat shock. *J. Dairy Sci.* **90**, 530.

Franco, M., Block, J., Jousan, F.D., de Castro e Paula, L.A., Brad, A.M., Franco, J.M., Grisel, F., Monson, R.L., Rutledge, J.J., and Hansen, P.J. (2006) Pregnancy rates in heat-stressed dairy cattle receiving one or two in vitro produced embryos in a timed embryo transfer program. *Reprod. Fertil. Dev.*, **18**, 202-203.

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Wrenzycki C., Block J. Herrmann, D., Niemann, H. and Hansen, P.J. Einfluss des insulin-ähnlichen Wachstumsfaktors-1 (IGF-1) auf das mRNA-Expressionsmuster entwicklungsrelevanter Gene in vitro produzierter boviner Blastozysten. Proc 34 Jahrestagung der Arbeitsgemeinschaft Embryotransfer deutschsprachiger Lander, p 2.